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VARIATIONS IN EUROTIUM HERBARIORUM, (WIGG.) LINK., INDUCED BY THE ACTION OF HIGH TEMPERATURES.

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Variations in Eurotium herbariorum, (Wigg.) Link. induced by the Action of High Temperatures.

BY

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With Plate XV and four Figures in the Text.

Introduction.

THE sudden variations shown by fungi in culture are familiar to mycologists, but evidence that such variations arise in wild material appears to be lacking. It is clear that the detection of variation in fungi on their natural substrata would not be easy, for the occurrence of a colony of unusual colour or form among a number of ordinary colonies would be interpreted as due to the presence of another species. If, however, a sector were seen in a colony of circular form, probably derived from one spore, there would be presumptive evidence that variation had taken place, though the demonstration would not be conclusive. Personal observation, extending over a number of years, of common saprophytic fungi on their natural substrata has not revealed a case of sectoring, nor has a mention of this phenomenon been found in the records of other workers. It therefore seemed possible that the variations recorded in cultured fungi might owe their origin to the technique used in the preparation of artificial cultures, or to the general conditions of culture. Attention was focused upon this possibility when variations made their appearance in a stock of *Eurotium herbariorum* which had remained constant in culture for five years.

Experiments with *Eurotium herbariorum.*

(1) *History of the strain used.*

Eurotium herbariorum was isolated in 1921 from a growth on the remains of a cigarette and, since that time, the stock has been maintained in culture on prune-cane sugar agar. Until 1923, the medium contained 40 per cent. of cane sugar; since then, 20 per cent. of cane sugar has been used. The change was made because difficulty was always experienced in inducing the more concentrated medium to set. The reduction in the

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amount of sugar has had no appreciable effect on the growth of the fungus. Until the autumn of 1925, the cultures were kept in an incubator at 30° C.; subsequent cultures have been grown at 30° C. From time to time, the purity of the stock has been assured by the transfer of single young colonies, but most of the cultures have been started from mass transfers of conidia, by means of a platinum wire.

A detailed history of the stock has not been preserved; cultures have been renewed at intervals of about two months, always on prune agar. When *E. herbariorum* has been required in quantity, the cultures have been made up in batches, and it is estimated that the total number of cultures made from this strain, under the ordinary conditions of culture, is about five hundred. All the cultures have been looked over, many of them in detail; some of them have been seen by a number of mycologists, others have been distributed to various laboratories. Consequently, this stock has come under the notice of many observers, and, had it shown a tendency to vary when grown on prune agar at moderately high temperatures, the fact could hardly have escaped notice.

In only one instance has a variation been noticed under ordinary conditions of culture. In the autumn of 1925 a pure white colony appeared among normal colonies in one dish; this form was isolated and appeared to agree with the conidial stage in all respects except colour. It was subcultured through five transfers, all on prune agar; growth became weaker and weaker, and the white form died out without yielding perithecia or developing any colour.

In 1926 cultures of *E. herbariorum* were prepared from the stock strain on a synthetic medium with the object of determining the lowest concentration of sugar necessary to the production of perithecia. In a given series of cultures all the transfers were made from one stock culture on prune agar, and in order to minimize the risk of contamination of the stock dish, which had to be opened a number of times, the infections were made as rapidly as possible. In one series of these cultures a light-coloured colony appeared in each of two dishes; these two dishes were the last of a series of thirty-six to be inoculated; they contained normal colonies as well as the abnormalities. Successful transfers were made from the light-coloured colonies, and these transfers formed the starting-points of three series of cultures (*A*, *B*, and *C*), which will be described in a later section of this communication.

(2) *Media.*

Throughout the work, stock cultures have been made on prune agar, and all the spores used for experiments have been taken from these cultures; all the stock cultures have been examined at frequent intervals and guarded against contamination.

In the investigation of the variants cultures have been made on prune agar, on a synthetic medium, and on Czapek's agar, as modified by Thom and Church (15).

The media used have been prepared as follows:

(i) *Prune agar.*

Twenty-five prunes are boiled for an hour; the liquid is poured off and made up to 1 litre; 400 grm. of cane sugar and 50 grm. agar are added.

(ii) *Czapek's agar.*

This medium was used in the modified form proposed by Thom and Church (15).

Distilled water	500 c.c.
Sodium nitrate	1·0 grm.
Dipotassium phosphate	0·5 „
Magnesium sulphate	0·25 „
Potassium chloride	0·25 „
Ferrous sulphate	0·005 „
Cane sugar	15·0 „
Agar	10·0 „

(iii) *Barnes's medium* (medium S.).

This medium has been found useful as a synthetic medium (Gwynne-Vaughan and Barnes (9)).

Distilled water	500 c.c.
Ammonium nitrate	0·5 grm.
Potassium nitrate	0·5 „
Tripotassium phosphate	0·5 „
Cane sugar	100·0 „
Agar	25·0 „

Media were sterilized at two atmospheres for twenty minutes, and poured into hot, freshly sterilized Petri dishes. As soon as the media were set, the dishes were transferred to an incubator at 35° C., and left for two days before inoculation. Enough dishes were prepared at a time to provide a complete experimental series; from these the dishes for the control cultures were chosen at random.

(3) *Experiments with heated Spores.*

The two abnormal colonies which were obtained in 1926 appeared on medium S., containing glucose in place of cane sugar, but, as they were accompanied by normal colonies, it seemed unlikely that they owed their origin to a direct effect of the medium; further, one hundred cultures had been made on this medium; they contained thousands of colonies, all normal for the medium.

On reviewing the technique employed in making up long series of cultures, it was realized that, at the end of a large number of transfers made in rapid succession, the platinum wire might be hot, owing to repeated sterilizations in a flame. It was found that in such circumstances the wire could be sufficiently hot to char the skin, when drawn between finger and thumb. This suggested the possibility that the variants had arisen from spores which had been heated strongly by contact with an unduly hot wire.

In order to test this, transfers were made from a stock culture to twenty-four plates of prune agar, by means of a wire which was well heated immediately before each transfer. Three controls were set up with a cool wire (see Table I).

TABLE I.

Hot Wire Cultures.

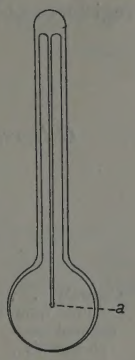
Medium. Prune agar. *Incubated* at 30° C. *Colonies* 5 days old.

Three controls	Colonies all normal
Hot wire cultures :	
In five cultures	Colonies all normal
In fifteen cultures	Some colonies normal, some abnormal
In four cultures	No growth

A detailed count of the colonies was not made ; it was estimated that each control contained from fifty to sixty colonies, that each hot-wire culture contained about forty colonies, and that the abnormal colonies amounted to about 8 per cent. of the total. The variations were slight, appearing as colonies a little more fluffy than usual, or with an enhanced crop of perithecia, or with a tendency to produce a slightly lobed margin. Subsequently, when stock cultures have been in course of preparation, an occasional culture has been inoculated with a hot wire, and variants have been obtained. From one of these Series E was started (p. 805).

Attempts were now made to build up a technique which would allow of better control of the exposure to heat, and, at the same time, make it possible to handle masses of spores without risk of contamination. Pieces of glass tubing of $\frac{1}{8}$ in. external diameter were prepared from carefully washed tubing, and, in order to provide a thin region which would break easily, a bulb of $\frac{3}{8}$ in. external diameter was blown at one end of each piece ; these tubes will be referred to as bulb tubes. The diameters of the parts were checked by passing them through holes of appropriate size bored in a piece of thick sheet brass. The bulb tubes were sterilized for fifteen minutes at 250° C., in clean sand, contained in a covered fireclay crucible. After cooling, the lid of the crucible was slightly raised, while a tube was removed with sterilized Carnot's forceps, and supported, mouth downwards, clear of the bench ; the lid of the crucible was immediately lowered.

Spores were at once introduced into the bulb tube by means of freshly prepared capillary tubing, of the diameter of a coarse hair, drawn from washed tubing. In order to facilitate rapid working, so necessary at this stage in order to reduce the risk of infection, a stock of capillary tubing, of approximately $\frac{1}{16}$ in. diameter, was prepared beforehand, and from this the thinner material was drawn. It was found that the fine capillary tubing was cool enough to use as soon as it was made, and it was then quite sterile. One end of the capillary tube was pushed among the conidia of a stock culture, then inserted into the bulb tube, and, by means of a small flame, sealed in position, so that the mass of spores hung in the centre of the bulb (Text-fig. 1).



TEXT-FIG. 1. Bulb tube ready for use. *a*. Position of mass of spores. × $\frac{4}{3}$.

For each series of experiments, enough bulb tubes were prepared to provide one tube for each dish to be inoculated, and a few additional tubes to allow for accidents, as the bulbs were very fragile.

The first exposures to heat were made by placing the bulb tubes, one at a time, for a given period in a thin-walled glass chamber surrounded by the vapour of boiling alcohol. After exposure, each tube was cooled by immersion in methylated spirit; this sterilized the outer surface. The tube was then washed in sterile water and broken into a dish containing prune agar. In this and subsequent experiments the control cultures were inoculated from tubes taken from the total number prepared for the series; these tubes were treated in all respects like those used for the experimental dishes, except that they were not exposed to heat.

TABLE II.

Cultures from Tubes exposed in a Chamber surrounded by Vapour of Alcohol. Temperature, 78° C.

Medium. Prune agar. *Incubated* at 300° C. *Colonies* 5 days old.

	Cultures made.	*Cultures showing no Growth.*	*Cultures containing—* *Normals.*	*Normals and Abnormals.*
Controls	11	—	11	—
Cultures from heated spores exposed for—				
1 minute	11	—	—	11
2 minutes	11	—	—	11
3 minutes	11	11	—	—
4 minutes	11	11	—	—

Abnormal colonies were distinguished by the development of low, white tufted outgrowths, which gave a rougher upper surface than is seen in normal colonies; there were indications also of a tendency to form many perithecia, and to produce loose fluff. Four transfers were made from

abnormal colonies to prune agar; the resulting colonies appeared to be normal.

A similar series was set up from tubes which had been exposed in the chamber surrounded by steam. The thermometer placed in the chamber registered 98° C. (see Table III).

TABLE III.

Cultures from Tubes exposed in a Chamber surrounded by Steam. Temperature of Chamber, 98° C.

Medium. Prune agar. *Incubated* at 30° C. *Colonies* 5 days old.

	Cultures made.	*Cultures showing no Growth.*	*Cultures containing—* *Normal.*	*Normal and Abnormal.*
Controls	6	—	6	—
Cultures from heated spores exposed for—				
30 seconds	7	—	7	—
1 minute	7	—	—	7
1½ minutes	5	—	—	5
2 minutes	7	—	—	7
3 minutes	9	7	—	2
4 minutes	5	5	—	—

Detailed counts of the colonies were not made. The control cultures contained from 30 to 50 colonies, those from spores heated for 2 minutes about 20 colonies; the two cultures which showed growth after an exposure of 3 minutes each contained 4 colonies.

The failure of these experiments to produce any marked variants suggested that sufficient heat had not been applied to produce effects comparable with those which could be afforded by a hot wire. It was resolved to expose spores to drastic treatment, and, in order that the experimental conditions could be repeated with some approach to uniformity, to heat the bulb tubes on molten lead which was just beginning to set. The spores were not expected to survive an exposure long enough to raise them to the temperature of the lead, but it was possible that the shock administered by a short exposure might be sufficient to influence the behaviour of the spores in a few favourable cases.

In the first series of experiments the bulbs were pushed into the lead for a given number of seconds, then immersed in spirit, washed in sterile water, and broken into a dish. The method was abandoned after one series, for not only did the lead form a coat over the bulb and hinder subsequent breaking, but it adhered so strongly to the glass that lead had to be placed in the culture. This introduced a new factor, and made necessary the introduction of small pieces of lead into the controls.

The results are given in Table IV.

TABLE IV.

Tubes coated with Hot Lead.

Medium. Prune agar. *Incubated* at 30° C. *Colonies* 5 days old.

	Number of Cultures.	*Cultures showing no Growth.*	*Number of Colonies—* *Normal.*	*Abnormal.*
Controls	5	—	230	—
Cultures from spores exposed for—				
1 second	5	2	10	—
2 seconds	7	3	13	—
3 seconds	7	4	7	2
4 seconds	7	4	7	—

A second lot of cultures were set up in which the bulbs were applied to the surface of the molten lead for a given period. In these cultures, there was no indication that metallic lead was transferred on the tubes. The experiments are summarized in Table V.

TABLE V.

Tubes placed on Hot Lead.

Medium. Prune agar. *Incubated* at 30° C. *Colonies* 5 days old.

	Number of Cultures.	*Cultures showing no Growth.*	*Number of Colonies—* *Normal.*	*Abnormal.*
Controls	—	—	320	—
Cultures from spores exposed for—				
3 seconds	7	—	33	—
4 seconds	7	2	21	
5 seconds	9	4	31	2
10 seconds	3	—	5	39
15 seconds	6	—	17	12
20 seconds	2	2	—	—

The production of normal colonies from spores set free from tubes which had been exposed to a high temperature for 10 and 15 seconds, and which were sufficiently hot to make a hissing noise when immersed in methylated spirit, suggested that the spores were not being exposed to heat in a uniform manner. It seemed incredible that the spores which germinated could have been exposed to a really high temperature; the patchy distribution of normals and abnormals indicated that the spores which germinated had been protected in some way. Possibly the air trapped among the spores prevented those within the mass from experiencing much increase in temperature; spores within the capillary tube might have been even better protected.

In order to bring the spores nearer to the source of heat, tubes of $\frac{1}{16}$ in. diameter, with bulbs $\frac{3}{32}$ in. diameter, were prepared. The small bulbs were

easily blown by sealing one end of a length of $\frac{1}{16}$ in. glass tubing, and then holding the other end in a hot flame. As soon as the end closed, it was transferred to a small flame, and the expansion of the air within the tube slowly blew a small bulb. The diameters were standardized by passing through holes in a piece of brass.

The results of a few trials with these small tubes are given in Table VI.

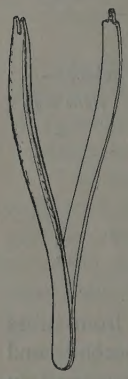
TABLE VI.

Small Bulb Tubes on Molten Lead.

Medium. Prune agar. *Incubated* at 30° C. *Colonies* 5 days old.

	Number of Cultures.	*Cultures showing no Growth.*	*Number of Colonies—* *Normal.*	*Abnormal.*
Controls	2	—	82	—
Cultures from spores exposed for—				
4 seconds	5	1	—	6
5 seconds	3	1	—	11

These results, though small in number, show a greater proportion of abnormal colonies than appears in the preceding tables. From this series the striking brown variant was obtained which is described in a later section under Series D (p. 804).



TEXT-FIG. 2. Forceps with tips shaped for breaking tubes. × $\frac{1}{2}$.

It was, however, felt that further efforts should be made to bring the work on to a more exact basis, although the methods employed had given some measure of success. Consequently, modifications were introduced. The bulb tubes had been used because the thin-walled bulb readily cracked with slight pressure from forceps; the preparation of a sufficient number of these tubes of standard size was, however, expensive of time. It was found that short lengths of capillary tube could easily be broken by a strong pair of forceps with the tips shaped as shown in Text-fig. 2, and that, by the use of these forceps, the risk of pieces of the tube jumping out of the dish at the time of breaking was much decreased. The tubes used were slightly under one millimetre in diameter, and were tested for size in the way already mentioned. One end was sealed, and the tubes sterilized and charged with spores in the same way that had been used for the bulb tubes. It was found advisable to seal the finer capillary into the outer one, for when the outer tube was broken the inner tube nearly always came out and brought spores with it; in this way, the chances of spores remaining inside broken pieces of tube, in positions unfavourable for germination, were lessened.

These small tubes were used in a series of experiments in which spores were exposed in a water bath for two minutes to temperatures ranging

from 49° C. to 98° C. After exposure they were treated in the way already detailed. It was hoped that these experiments would give some information leading to a more precise understanding of the course of events when spores are heated. The results appear in Table VII.

Table VII.

Tubes heated in a Water Bath.

Media. Prune agar (Pr.); Synthetic (S.) Incubated at 30° C. *Colonies* 6 days old.

Medium.	*Temp. in* °C.	*Number of Cultures.*	*Number of Colonies—* *Normal.*	*Abnormal.*
Pr.	Controls	6	199	—
S.	Controls	3	157	—
Pr.	49	3	116	—
,,	51	3	92	—
,,	52	3	20	—
,,	54	3	17	2
,,	55	3	54	2
,,	56	3	18	—
,,	58	3	—	31
,,	60	6	5	36
,,	62	2	2	8
,,	64	3	45	19
,,	66	3	17	12
,,	68	3	27	31
,,	70	3	17	19
,,	72	3	11	28
S.	74	2	5	3
,,	76	2	3	4
,,	78	2	12	1
,,	80	2	14	13
,,	84	2	18	5
,,	88	2	—	2
,,	92	2	—	3
,,	94	2	7	1
Pr.	96	3	—	—
,,	98	3	—	—

It is to be noted that in two of the controls on prune agar shown in this table parts of the tubes flew out of the dishes when they were broken. As a result, these cultures contained a small number only of colonies.

Table VIII.

Summary of Experiments with heated Spores.

A. Experiments in which colonies were not counted.

	Number of Cultures.	*Cultures giving no Growth.*	*Cultures containing—* *Normal only.*	*Normal and Abnormal.*
Controls	20	—	20	—
Cultures from heated spores	108	38	12	58

B. Experiments in which colonies were counted.

	Number of Cultures.	*Cultures giving no Growth.*	*Number of Colonies—* *Normal.*	*Abnormal.*
Controls	23	—	988	—
Cultures from heated spores	134	29	644	292

All the cultures from which this evidence was obtained were free from contamination at the time when they were inspected.

It is clear that the methods employed will give colonies differing to a greater or less extent from the stock strain of *E. herbariorum*, but the methods are erratic in operation, for cultures prepared in exactly the same way do not give uniform results.

A satisfactory method of working with single spores has not been devised, for the necessary manipulation greatly increases the chances of contamination of the cultures; in work of this kind a culture which is in any way suspicious cannot be accepted as evidence.

Efforts were made to secure a more even exposure of the spores to heat by using suspensions of the conidia in sterile water at known temperatures. It was found that the spores were readily killed by moist heat. In two sets of experiments, each including twenty-four cultures on prune agar, germination was obtained after conidia had been exposed for one or two minutes to temperatures of 48°, 50°, and 52° C., but germination could not be obtained after exposures to 54° C. and over. At 52° C. most of the spores were killed. The controls, and the cultures in which any germination occurred, gave normal colonies exclusively.

The behaviour of the ascospores has not been investigated. The stock strain of *Eurotium* forms conidia freely, so that it is difficult to isolate a perithecium free from conidia. The microscopic examination necessary to ensure this involves a good deal of handling, and so introduces a serious risk of contamination. Twelve cultures have been made from perithecia believed to be free from conidia; in all cases these cultures contained colonies of *Penicillium* within two days of setting up, and were discarded.

Of the considerable number of aberrant colonies which have been observed, most differed but little from the normal form; in many the variation was apparent only up to the time that the colony was six to seven days old; later, the peculiar features often disappeared, so that the colony could not be distinguished from a normal colony of the same age. In general, the differences consisted in slight variations of colour, in the width of the margin of the colony, and in a tendency to produce upstanding hyphae from the surface, giving roughness or fluffiness according to the length of the hyphae and the abundance of their production. Rather more obvious changes occurred in colonies which produced an unusually heavy crop of perithecia; in a few cases the alteration was so great that the fungus did not look like *E. herbariorum*. In estimating whether or no a given colony was abnormal, attention was paid to the effects of crowding in causing a change of appearance; it was usually possible to compare any colony with one similarly situated in a control culture.

The effect of exposure to heat was seen not only in the variant colonies produced, but also in the germination of the spores. It is to be

remembered that all the tubes for a series of cultures were charged with spores before any were heated or broken into a culture dish, and that it was purely a matter of chance which tubes were used for controls. In the experiments the control cultures developed an average of forty-three colonies, the cultures from heated spores an average of seven colonies. Evidently much killing occurred. Evidence was obtained that heating the spores delayed germination, and led to the development of slowly growing colonies; development was more rapid in the controls than in the other dishes of a series, even when variants did not appear. When normal and abnormal colonies occurred in the same culture, the abnormals often arose from retarded spores, and grew slowly at first. Experience showed that variants might be expected in dishes which contained few colonies; the association of a high death-rate with the appearance of abnormal colonies is significant.

A large number of variants has been obtained in these experiments. It has been impossible to make subcultures from them all, but series of cultures have been obtained from the more striking variants.

Description of the Variants.

Eleven of the variants obtained were well defined; to these arbitrary names have been assigned. They were suggested by the appearance of the colonies, and have been found useful labels. Some of the variants have been matched with published species, but, as a complete morphological and systematic study has yet to be made, the fanciful names are retained here.

As a basis of comparison, a brief account is given of the manner of development of the stock strain of *E. herbariorum*, grown on prune agar at 30° C. Germination is apparent to the naked eye about twenty-four hours after sowing. The young colonies are somewhat fluffy, white, and loose. By the end of the second day the colonies may have a diameter of 1 cm. or more; their centres are occupied by a loose turf of green conidial heads, with white and younger heads surrounding them. By the end of the third day the appearance of a yellow colour in the centre of the colonies indicates the beginning of perithecia. Subsequent events depend a good deal on the amount of room available. When the colonies are crowded, the centre of each is usually occupied by a circular area covered by conidiophores, and surrounded by a region of looser growth in which perithecia abound and conidiophores are relatively few. When single spore cultures are made, or when few conidia are transferred to a dish, the dense conidial central area may not form, and a culture a week old may show perithecia scattered all over the surface. Except when conidiophores form in crowded patches, the surface of the medium is not usually completely obscured, and the perithecia are not close together; in old cultures the hyphae do not form a conspicuous feature. When incubation is carried on at temperatures

between 37° and 42° C. the production of perithecia is usually increased, and that of conidia diminished; at these temperatures dense, unbroken yellow areas may be obtained.

A normal culture a week old, and still in process of growth, is commonly of the following colours:[1] conidial centre, ivy green (XXXI, 25″, m.); perithecial region, lemon yellow (IV, 23, – and b); growing fringe, shading through glaucous greens (XLI, 33‴, i, –, b, d, f) to white. The colonies are circular in outline, and the medium is not stained.

Divergences from this normal form are shown by the variants in the shape of the colony, in colour, in the relative proportions of the crops of conidia and perithecia, and in the reactions to the medium, as shown by the production of stains; changes in morphological characters have also been noted. It has been shown, by the preparation of mixed cultures, that the differences existing between the normal form and the variants, and between the variants themselves, are not due to the medium, for in such cultures each type shows its peculiar features.

The Principal Variants.

(1) *Flame* (Pl. XV, Fig. 2).

Flame is the characteristic variant of Series A. It has been obtained only in this series, though an approach to it was seen in an early culture of Series B, and something resembling it was obtained in a mixed culture derived from spores heated to 76° C. On prune agar growth is recognizable in twenty-four to thirty-six hours after sowing. The young colonies show a thin prostrate growth of radially arranged hyphae, and at an age of two to three days the development of a faint green tinge in the centre of each colony accompanies the formation of the conidiophores. Soon afterwards, the centre of the colony becomes bright yellow by the pigmentation of the hyphae, which form a low but dense covering over the medium; as the colony spreads, the yellow area extends, whilst the fringe is occupied by a small number of conidiophores which soon collapse. A colony five to six days old shows a bright yellow centre (III, 17, –), passing into lemon yellow (IV, 23, b), and then into the green peripheral region (XLI, 29‴, f, and 33‴, f); the green is due to the conidial heads. In from ten to fourteen days, red-brown islands (II, 9, k) appear in the central yellow; these islands spread and darken, until, in about a month, the whole colony is burnt sienna (II, 9, k) or vandyke brown (XXVIII, 11″, m) in general colour; the whole surface of the medium is now covered by a felted mass of hyphae containing a few scattered perithecia; these are yellow or orange (IV, tones in horizontal lines b). This appears to be the definitive facies.

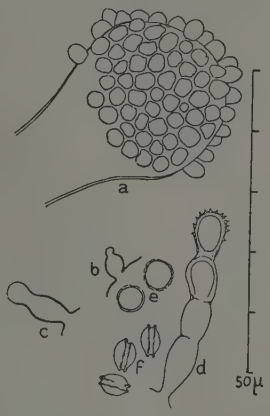
On the synthetic medium similar colonies are formed, but the reds

[1] In this, and in succeeding descriptions, colour matchings are based on Ridgway's Colour Standards and Colour Nomenclature (vii). The manner of reference is that used by Ridgway.

and browns tend to be brighter. Satisfactory growth has not been obtained on Czapek's agar.

Examination shows that the development of the red and brown colours is due to the accumulation on the hyphae of scales of secreted material. These scales dissolve in glacial acetic acid, to form a red solution; they are difficultly soluble in spirit, and apparently insoluble in water. Treatment of the hyphae with 50 per cent. hydrochloric acid gives a reddish tint, which is converted to a reddish purple by the addition of excess of ammonium hydroxide.

The colonies of this variant are seldom regular in outline; in general, the margin shows blunt capes and wide bays, which impart a characteristic form to the whole. On a few occasions, however, a subvariant has been obtained which forms circular colonies, a relatively heavy crop of conidia, and does not darken in colour beyond the burnt sienna stage.



TEXT-FIG. 3. Details of the flame variant. *a.* Young vesicle at apex of stalk of conidiophore. *b, c.* Stages in development of sterigmata. *d.* Chain of young conidia. *e, f.* Ascospores in face view and in side view.

The following morphological details of 'flame' have been obtained:

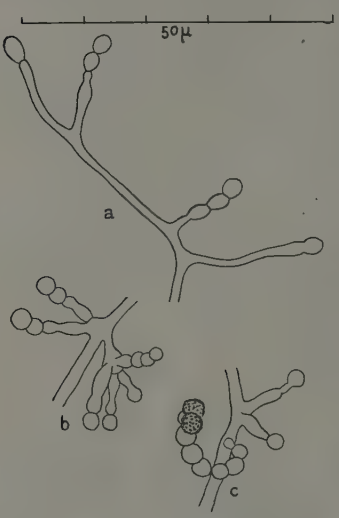
Conidia ovoid, 7×4–$5\,\mu$; not showing much variation in size; young conidia smooth, mature conidia rough, with bluntish outgrowths from the walls; conidia greenish in mass, almost colourless singly. Sterigmata in one series, crowded, springing from the upper three-quarters of the vesicle; stout, drop-shaped, with a blunt apex, $7{\cdot}5$–8×3–$4\,\mu$. Vesicles rounded, 20–30 μ in diameter; averaging $23\,\mu$. Stalks usually aseptate, occasionally with a septum close to the foot cell, and rarely with a septum just below the vesicle; stalks showing a gradual increase in diameter from below upwards ($6{\cdot}5$–$7\,\mu$ below, 9–12 μ at the junction with the vesicle). Walls of stalk smooth, slightly thickened; thickening of almost even amount throughout. Stalks 300–400 μ long.

Perithecia resembling those of the normal strain, globose, pale yellow or orange, 60–80 μ in diameter. Ascospores averaging $7 \times 5\,\mu$, distinctly but not deeply furrowed, and with slight development of a frill (Text-fig. 3).

(2) *Green flame* (Pl. XV, Fig. 3).

Young colonies resemble young colonies of flame, but within three days of sowing the colonies of green flame are distinguished by their greater fluffiness and heavier crop of conidia; at this stage the colonies might be mistaken for the normal form. The green central region shows

tones ranging from greenish glaucous to American green (XLI, 33‴, f–i.). Limited crops of perithecia develop beneath the fluff and impart a dull yellow colour to the margins of the colonies. Old cultures show a fairly even coating of dark green conidia (XLI, 33‴, k, m), with yellow speckling due to groups of perithecia, and slight browning of the hyphae occurs. The ascospores may be without frill or furrow, and either with a faint polar flattening or fully ellipsoidal. Green flame differs from flame in the production of heavier crops of conidia, and in the absence of bright red and brown incrustrations on the hyphae; it diverges from normal in the greater fluffiness and in the relatively poor crop of perithecia.



TEXT-FIG. 4. Formation of conidia on the aerial mycelium of the creamy variant. *a*. Simple chains of conidia. *b*. Small vesicle with sterigmata and conidia. *c*. Older simple chain, showing twist.

(3) *Woolly flame* (Pl. XV, Fig. 4).

Woolly flame is a strongly growing form, characterized by a tendency to produce zones of long conidiophores. When two days old, a colony consists of a loose hemispherical mass of radiating white hyphae. After three days, the colony may be 3 cm. in diameter, with a dense tall central area occupied by crowded conidiophores and a peripheral ring of radiate hyphae, which are white, and may attain a length of over a centimetre. Later, the development of perithecia in the centre is indicated by the appearance of bright yellow colours (IV, 19, –, and III, 15, –), and as this coloured centre extends one or more zones of long conidiophores may be formed outside it. As the colonies age, the long conidiophores collapse and lie on the surface of the medium; eventually, the conidia may become almost black in mass.

(4) *Rich conidial.*

The young colonies of this variant resemble those of No. 3. Later, conidia form in large numbers, often in more or less distinct zones; in mass the conidial heads are ivy green (XXXI, 25″, m.). Perithecia are scantily produced. Old cultures do not show browning of the hyphae.

Variants 1–4 are characterized by the production of rather small crops of perithecia. Woolly flame and flame produce much aerial mycelium; in the former it is loose, in the latter felted; both show bright colours in the

hyphae around the perithecia. Green flame resembles flame in the browning seen in old cultures, but in its general characters approaches the normal form; rich conidial is still closer to normal. Variants 2–4 may be oscillations between flame and normal; of them, green flame alone is fairly constant; subcultures from the others may give the parent form, but often give green flame, and sometimes forms which are not far from normal.

(5) *Blue conidial* (Pl. XV, Fig. 5).

This variant was obtained in Series A, and independently in Series H. It differs from the variants already described in forming heavy crops of perithecia and, often, relatively few conidia, which are glaucous green (XXIX, 3), but appear blue against the dense yellow background of perithecia.

Young colonies are low and colourless, and resemble young colonies of flame; they are, however, distinguished by a streaky appearance. By the fourth day, many perithecia are present, and a small conidial region occupies the centre of the colony. The perithecial region is usually lemon yellow (IV, 23, –); it may remain so, or in old colonies turn towards cadmium yellow (III, 17, –) or ochre (XV, 17, –). The radial streaking seen in young colonies persists, and in old and large colonies the radial arrangement shows in a deeply lobed margin.

On Czapek's agar the variant gives dense colonies which grow slowly and bear low whitish fluff among the perithecia. These colonies look much like colonies of the normal form grown on this medium, but they differ in the secretion into the medium of a brown stain (XXIX, 13″, i). This staining effect separates the blue conidial variant from the normal form when grown on this medium in pure culture, but when the normal and *Penicillium* are grown together on Czapek's agar, the former yields the brown stain. A similar staining effect has been obtained on Czapek's agar from the progeny of spores heated to 92° C.

The variants which are now to be described are regarded as being further removed from the normal form than are the foregoing. They have shown less tendency to vary in culture, and are apparently more stable in constitution.

(6) *Creamy* (Pl. XV, Figs. 6, 7, 10).

The name 'creamy' was suggested by the appearance of young colonies of this form. Growth is slow, and three days after infection the colony is a small white hemispherical tuft, 2 to 5 mm. in diameter. As growth continues, the colony becomes denser, and assumes the form of a whitish cushion tinged with a creamy flush. By the sixth day, green can be seen beneath the white coating of hyphae covering the centre of the colony. Later, the centre may become definitely green (XVII, 29′, k; XLVII,

25′′′′, –; or XLVII, 29′′′′, d), due to the emergence of the conidiophores from the fluff, or it may spread widely and retain its white or yellow fluffy character. The dense form and slow growth of this variant reached an extreme expression in one culture which did not produce conidiophores, but grew slowly as a pinkish white cushion and stained the medium a deep maroon (I, 3, m).

A complete morphological study of the creamy variant has not been made. The peculiar conidial branches and imperfect heads which develop on the fluff are shown in Text-fig. 4 (p. 796).

This type has been obtained in Series A, B, C, E, and P.

(7) *Dense orange.*

Throughout the work with creamy in Series B, colonies were obtained which showed a good deal of yellow and orange in the fluff, and occasionally small colonies of a dense fluffy orange form were seen. For some time, transfers from these colonies failed to give the orange form in pure culture. It has now been isolated, but it cannot be said definitely if it will persist, though it has been obtained pure in five series of transfers, comprising fourteen cultures. The colonies are dense and cushion-like, coloured in orange and yellow (III, 17, b to IV, 21, b); as they age, the orange colour becomes more or less hidden by a heavy development of white felted hyphae.

(8) *Sea-green.*

The sea-green variant was obtained in Series A, B, C, and E, in cultures made from creamy colonies. Young colonies are white, dense and fluffy, like those of creamy, but they soon become green (XLVII, 29′′′′, b; XLVII, 33′′′′, b and d), and spread over the medium as loose, often zoned colonies with a definite white margin; these colonies have a superficial resemblance to *Penicillium*. Sea-green has not shown a tendency to produce orange colonies, but occasionally yellowish green (XLVII, 25′′′′, –) colonies have been obtained, and in both Series A and B colonies have appeared which secrete a brown stain into the medium.

On Czapek's medium, both creamy and sea-green give rise to extraordinary heaped, fluffy colonies, at first white, later flushed with cinnamon, and, after about a month, spotted with grey tufts (LII, 35′′′′, –) due to the emergence of sheafs of conidiophores. On prune agar both variants appear to be fairly constant in character, though it seems as if the dense habit of growth characteristic of creamy is being lost, and that sea-green is the more stable form.

(9) *Speckled* (Pl. XV, Fig. 8).

This variant seems to approach closely to *Aspergillus nidulans*, and in particular to No. 4110 described by Thom and Church (15). It was obtained from cultures of creamy on Czapek's medium, and since it has been

in culture has shown little tendency to vary. On prune agar growth is rapid; young colonies show long whitish hyphae arranged on the medium in a bent radial manner; soon they are obscured by an abundance of olive-green conidia (XLVII, 29′′′, i); in four to six days small yellowish spheres, 0·5–1 mm. in diameter, make their appearance; these consist of hollow masses of globular, thick-walled cells, the Hulle cells as described by Eidam (7), surrounding perithecia containing masses of purplish brown ascospores.

(10) *C yellow.*

This variant has been found in Series C only, and has remained constant in character. Young colonies are low, almost colourless, later whitish, and by the fourth day, when the colonies are 1·5–2 cm. in diameter, faintly yellow (IV, 23, f) in the centre. About this time, hyphae begin to grow a little beyond the general margin of the colony and to produce tufts of small hyphae lying on and in the medium; they cause the development of a characteristic lobed margin. The colonies cover the surface of a 10 cm. dish in about a fortnight. An old colony is nearly uniformly yellow (IV, 23, b), and the medium is usually rather loosely covered. Conidiophores are never a conspicuous feature of the cultures, and the crop of conidia is low. Perithecia are irregular in outline, but are not comparable with the compound perithecia recorded by Fraser and Chambers (8). Giant cells, resembling those described by Wehmer (16), formed by *A. fumigatus* in acid media, develop within the substratum.

11. *D Brown* (Pl. XV, Fig. 9).

D brown was obtained as the only colony which developed from a tube of spores heated on molten lead on March 1, 1927; it is perhaps the most striking form obtained in this work. On prune agar young colonies appear as hemispherical groups of pure white hyphae, radiating from a common centre: two days after inoculation, the colonies have a diameter of 4–6 mm., and in four days, by which time the colony has become cushion-shaped, a brown colour beneath the white fluff indicates the presence of conidiophores. At this stage, apart from the difference in colour, the colonies resemble closely those of the creamy variant. In the absence of crowding, a spreading, zoned colony is formed; the centre is occupied by a cushion of brown conidial heads (XXVIII, 11′′, m.), and the rest of the colony consists of alternating zones of sterile hyphae and conidiophores; crowding suppresses zonation. When the colonies are 12–14 days old, a dense white layer of hyphae forms under the fluff, over the surface of the substratum: it is most clearly seen in the relatively clear areas between the zones of conidiophores. The layer thickens, changing in colour through buff (XXX, 19′′, f.) to honey yellow (XXX, 19′′, –), and in cultures two to three months

old may be 1–2 mm. thick, and of a consistency recalling that of the yolk of an hard-boiled egg. It contains enormous numbers of sausage-shaped or coiled Hulle cells. Perithecia have not been found, although cultures up to five months in age have been examined. On the synthetic medium development is much as on prune agar, but the production of Hulle cells is greatly increased. On both media old colonies give rise to a good deal of cobwebby fluff.

On Czapek's medium growth is slow and dense; the colonies do not show definite zonation, and the conidial heads may appear almost black.

The conidiophores of this variant are 250–500 μ in length; stalks often slightly sinuous, and sometimes forked, 4–6 in diameter in the widest part, and tapering slightly from this towards base and apex; vesicles subclavate, 10–12 × 12–15 μ, arising by a rather abrupt swelling of the stalk. Walls of stalk smooth, evenly thickened, and faintly brown, particularly below the vesicle. A definite foot cell is present, distinguished from the rest of the hypha by the thickening of its walls. Sterigmata in two series, arising from the greater part of the vesicle, or in a single series. Conidia globose, with blunt roughenings on the surface, 3–5 μ in diameter, chains of conidia often adhering to form several columnar masses radiating from one head. In addition to the ordinary heads, small ones are developed from the hyphae of the fluff. These heads are usually without a vesicle; sterigmata may arise in groups of three or four from the end of a short branch, or in small groups in succession from the same branch.

The Behaviour of the Variants in Pure Culture.

Series of cultures have been made from the two aberrant colonies which suggested this investigation, and from abnormal colonies afterwards obtained from spores intentionally exposed to heat. Care has been taken to make the transfers with a cool wire. The cultures have been kept in an incubator at 30° C.

Series A and B.

On November 15, 1926, a dish containing the synthetic medium, but with glucose in place of cane sugar, was inoculated with conidia from a stock culture of *E. herbariorum* on prune agar; this culture was about a month old and showed no abnormal colonies. On November 18, 1926, in addition to normal colonies, a whitish colony was noticed, and on the 20th it was clear that this colony bore heads of *Aspergillus*-form; at this time the normal colonies bore conidia and perithecia. On the 22nd, transfers of conidia were made from the whitish colony to two dishes of medium S, with glucose in place of cane sugar; these cultures were the starting-points of Series A and B respectively.

Series A.

A single spore culture was not made in the early cultures of this series. The first cultures were made on the synthetic medium, and gave whitish colonies like the parent. On January 14, 1927, a transfer was made to prune agar, and another to the synthetic medium; on the prune agar a fluffy colony was formed, which later became a dense, low, felted cushion showing a mixed coloration of browns, pinks, and yellows; on the synthetic medium the growth was whitish. Five more cultures were made in succession on the synthetic medium. In March 1927 conidia were transferred to prune agar from the culture of the 14th January, and from the youngest of the cultures on the synthetic medium; the parent cultures were clean. Densely felted colonies were again obtained, both showing some green. The work on this series has been continued from these cultures, and the series has been split into two sections, one characterized by the flame variant and its derivatives, the other by the creamy variant. With respect to the latter, some cultures have been prepared in order to find if the creamy of this series would differ in behaviour from the creamy of Series B, which was being cultured at the same time. In Series A the creamy variant has not shown a tendency to give rise to orange colonies, but with this exception it has behaved much as in Series B. A full discussion of the behaviour of this variant is therefore reserved for the account of that series.

It was realized that prune agar is variable in composition, and it was considered necessary to introduce a synthetic medium of constant composition into the work. Accordingly, plates of Czapek's medium were prepared and inoculated; this was done not only in Series A, but in other series also. The results were disappointing, for slowly growing colonies were obtained which gave dense felted cushions (Pl. XV, Fig. 7) in which the characteristics of the variants, as developed on prune agar, could not be made out. The colonies were at first white, later they became flushed with tones of cinnamon, and later still, after the production of copious drops of a vinegar-coloured exudation, they gave rise to tufts of greyish conidiophores. Cultures on Czapek's medium inoculated with conidia from stock cultures gave colonies which were recognizably *E. herbariorum*, though the growth was dense; these cultures brought out a clear difference between the aberrant forms and the stock material. The culture of flame on Czapek's medium, in which growth was very poor, was opened for examination, and so was not used in subsequent work; from the cultures of creamy on Czapek's agar creamy was obtained again on prune agar.

As Czapek's medium appeared unsuitable, recourse was had to medium S; it was known that *E. herbariorum* retained its characteristic features on this medium, although the growth was denser than on prune agar.

Conidia were transferred from the variants to plates of medium S, and

corresponding transfers were made to prune agar. The variants grew more strongly on the synthetic medium, but they retained their peculiarities. A second set of cultures was therefore set up, and it was hoped that the work could be continued exclusively on a standardized medium. However, it soon became clear that the use of the synthetic medium must be discontinued, for new variants appeared in cultures of flame, inoculated with conidia from cultures on that medium. It would have been interesting to have continued to use this medium, as further variants might have been obtained, but it was necessary to limit the bounds of the work, which was becoming unmanageable. Moreover, it was evident that the use of the synthetic medium was introducing a fresh complication, as it could not be decided at the time whether the new variants were an expression of instability in the flame variant, or entirely due to the effect of the synthetic medium. Consequently it was resolved to use prune agar for subsequent work; this had the further advantage of retaining in use a medium on which the behaviour of the stock strain of *Eurotium* was well known. The new variants, such as blue conidial, green flame, woolly flame, and rich conidial, have retained their characters with some degree of consistency on prune agar. This is also true of the flame variant, which has remained almost unchanged throughout the series of transfers from prune agar to prune agar, though slight variations have been noted in the crops of conidia and in the form of the margin.

The behaviour of the creamy colonies of Series A, after a period on medium S, was not investigated.

At a later stage in the work, some cultures were prepared on Czapek's medium, in order to test again the behaviour of the variants on this medium. Blue conidial gave dense yellow colonies with brownish conidia; when the culture was eight days old, the medium was stained mars brown (XV, 13′, m). Similar colonies have been obtained from spores heated to 92° C. in Series W. Green flame gave rather poor colonies in which the characteristics of this variant were indistinctly seen. Flame gave poor growth, and woolly flame was not tested. Evidence has not yet been obtained that Czapek's medium will favour the appearance of new variants in this series.

In a few cultures of Series A, after the variants had been passed over medium S, colonies were obtained which approached closely to the normal form in colour and habit; they differed, however, in marked looseness of growth, or in the production of a polygonal pattern owing to the strong aversion between the colonies. Similar cultures have been obtained directly from heated spores.

In one instance, flame was transferred to a plate of prune agar which had been incubated at 40° C. for a month previous to sowing. On this relatively dry medium, low, dull green colonies resembling poorly grown sea-green appeared, and from these sea-green was obtained on ordinary

prune agar. This solitary observation may indicate a change in behaviour due to the dry medium, but it is inadequate as a demonstration.

A review of the work on Series A suggests the following comments:

1. In early cultures of the series, the colonies tended to develop a dense felted growth of mixed coloration. As a single spore culture was not made at this stage, the peculiar growth may have been due to a mixed population. It has not, however, been possible to produce such a growth by sowing the spores of two or more variants together. In such cultures the variants develop separately, and show their usual characters.

2. Once a variant is isolated in pure culture, it retains its characteristics with little change, as long as it is grown on prune agar. It is probable that such variations as do occur are mainly due to slight variations in the composition of this medium.

3. A variant form may be induced to vary further if grown on medium S and subsequently transferred either to the synthetic medium or to prune agar. Variants obtained in this way from the flame variant tend to approach normal *E. herbariorum*.

Series B.

The origin of Series B has already been described (p. 800). On December 11, 1926, a transfer was made to prune agar, and on the 14th a single colony was cut out from this culture and placed on prune agar. A dense, fluffy, felted colony grew slowly. As in Series A, there has been a general tendency, as successive sets of cultures have been made, for the colonies to become less felted and looser in growth. At first, creamy colonies predominated; later, sea-green was more abundant, though it is sometimes difficult to determine if a colony should be described as loose creamy or dense sea-green.

Throughout the investigation of this series colonies have been obtained which showed clearly the presence of a yellow pigment. At times the white margin has contained suggestions of yellow areas; sometimes the margin has been yellow; occasionally colonies of creamy form, but entirely yellow or orange (the dense orange variant), have appeared, mingled with creamy colonies. The orange variant has been isolated, and shows signs of constancy. In an early culture of the series the young colonies were definitely creamy, but as the culture aged scattered patches of orange formed in the fluff, and from the progeny of this culture colonies of flame developed; it seems likely that the orange patches were an indication of the tendency which later gave the flame variant in this line.

Evidence was not obtained in Series B that the appearance of new variants could be induced by the use of medium S. On the other hand, changes seem to have been favoured by the use of Czapek's medium. The

striking 'speckled' variant which first appeared on July 11, 1927, was obtained in a transfer from a culture on this medium, and the strong tendency shown by some cultures to produce orange colonies may be due to the same cause.

In one culture, also derived from a culture on Czapek's medium, three colonies, which seemed just like their neighbours in form and colour, secreted a brown stain into the medium, and the same phenomenon was observed in transfers from this culture. The most extraordinary staining was shown by a culture of Series B on medium S. The colony grew very slowly, for a long time remained white, and eventually assumed a delicate pinkish colour; it secreted a port wine stain in abundance. These staining reactions suggest that, although the fungi of Series B show less morphological variation than was found in Series A, yet they too are unstable in constitution.

The variants of Series B grow relatively slowly, often form a strong aerial mycelium in the form of dense felted fluff, frequently stain the medium strongly, and, with the exception of the speckled variant, do not form perithecia. The creamy variant has been obtained in Series A and C, and also in Series E and P.

Series C.

The colony which gave rise to Series C developed among normal colonies in a companion culture to that which gave Series A and B. From the early cultures of the series the C yellow variant and the creamy variant were obtained; both have been maintained in culture. C yellow has shown little variation in culture; it does not seem to react to a change of medium. It remains recognizable on Czapek's medium, though growth is weak and old colonies turn brown.

C yellow is the only variant which has shown sectoring.[1] In one culture four sectors of the normal form were developed, and from these the normal form of *E. herbariorum* was isolated.

Series D.

The colony which formed the starting-point of Series D was the only colony obtained from a tube of spores of *E. herbariorum*, heated for four seconds on lead, on March 1, 1927. Transfers from this gave the D brown variant.

As in Series C, very little variation has been seen in the behaviour of the cultures in this series. There have been slight variations in the extent of zonation, in the strength of development of the Hulle, and in the amount of aerial mycelium, but these differences seem capable of explanation on spatial relations; when the colonies have plenty of room, zonation is usually strong; when they are crowded, a central conidial tuft arises, but zonation

[1] Since this was written, sectoring has been obtained in blue conidial, with the production of a subvariant.

is suppressed. Transfers of conidia to medium S and to Czapek's medium have yielded colonies which showed growth and coloration unlike those seen on prune agar, but when transfers were made to prune agar the original form reappeared, or slightly modified colonies were obtained. The D brown variant appears to be nearly constant, and not liable to marked variation.

Series E.

On June 14, 1927, four transfers of conidia were made to prune agar with a cool wire, and one with a hot wire; all were made from the same stock culture. The four developed normally, with forty to fifty colonies in each; the fifth bore ten normal colonies, and a flat cushion of creamy type, with a pinkish white margin. A transfer was made to prune agar, and colonies were obtained, very like the usual creamy colonies, but with a yellow margin; as these colonies developed, radiating cracks formed in the fluff above the centre, and through these the green conidial area was seen. The yellow margin and the radial fissuring indicated that this variant was not the same creamy type as that of the earlier series. The investigation of this series has revealed nothing of special note; it has yielded creamy and sea-green.

Series H.

On July 21, 1927, conidia of *E. herbariorum* from a stock culture of June 4, 1927, were heated in a capillary tube for two minutes at 60° C. Three colonies were obtained in the culture prepared from this tube, all with few conidia and many perithecia; from the most abnormal of these a transfer was made to prune agar. A low colony was obtained, ochre (XV, 15′, b) in colour, showing faint signs of zonation, and bearing a poor crop of brownish conidia. As successive cultures have been made in this series the production of conidia has increased, and the later colonies of the series present characters intermediate between those of the blue conidial type of Series A and normal *E. herbariorum*.

Other Series.

In addition to the series just described, ten other isolations have been made from abnormal colonies obtained from heated spores. The creamy variant, differing slightly from that of Series B and E, was obtained in Series P; once recognized, this was not retained in culture. In the other series the variations were less marked. These series have yielded colonies of unusual form, which resembled some of the colonies obtained in Series A; thus, in Series F and V, colonies of low growth and strong mutual aversion have appeared, and in Series V an approach to the flame variant has been seen in colonies producing perithecia among bright orange fluff; this series has also yielded a form very like green flame, but with the conidia almost black. Dense yellow colonies, staining Czapek's medium brown, have been

obtained in Series W; in Series Y and Z, colonies normal in most other respects have formed the lobed margin characteristic of C yellow. In Series L colonies have been obtained which yield many perithecia, much yellow mycelium, and very few conidia; these colonies resemble closely those produced by the stock form at temperatures above 40° C., and are not stable. All these series have been started from spores heated in capillary tubes in water, at temperatures between 60° C. and 94° C.; all have yielded definite abnormalities, but none has been carried through many transfers.

The Behaviour of the Variants in Mixed Culture.

During the progress of the work variants have been grown in mixed culture, with other variants, with the normal form, and with *Aspergillus niger*. Little of note was obtained with the mixed variants, but some interesting observations were obtained with the other cultures. The creamy variant grown by itself on prune agar retains its dense, fluffy, whitish or yellowish margin; when grown with the normal form it shows a noticeable greening of the margin, and when grown with *A. niger* the greening is pronounced. The change is due to the formation of an increased crop of conidial heads. On Czapek's medium the association of creamy and *A. niger* gives nothing of note. In pure culture on prune agar, D brown retains its brown conidial heads, or maybe, in old cultures, develops tardily a few greenish heads in the central tuft. When this variant is grown with *A. niger*, both planted at the same time, the development of the D brown colonies is limited by the more rapid growth of *A. niger*, and small colonies, with green conidia only, appear. The inoculation of *A. niger* into a dish containing a well-developed colony of D brown causes distinct greening of the latter as soon as the two fungi approach one another, owing to the development of green conidial heads, which tend to hide the older brown ones. On Czapek's medium D brown stains a pale yellow on the underside of the colony; in pure culture *A. niger* does not produce any appreciable staining; when the two are grown together on Czapek's medium D brown stains pale yellow, and *A. niger* secretes a diffuse blackish stain into the substratum. Speckled fails to produce perithecia in contact with *A. niger*. Flame and C yellow do not seem to react to the presence of other fungi.

Discussion.

In the course of this investigation many fungi with conidial heads of *Aspergillus*-form have been isolated in pure culture; some of these forms have shown constancy under the conditions offered to them, others have varied when the composition of the medium has been altered. Some have retained their characteristics through a long series of transfers, others have persisted for a time and have given rise to new variants. It seems evident that the

variants owe their origin to the effect of heat on the spores, but the experimental results and the conditions under which the fungi have been grown have been reviewed, in order to determine if any other cause could have produced the effects observed.

It might be suggested that the variants are due to the effect of a change of medium on the fungus; Brown (5) showed that saltation was encouraged in *Fusarium* by the use of a concentrated synthetic medium. Evidence has been obtained in *Eurotium* that the behaviour of the variants may be changed by a change of medium, and that new forms possessing some degree of constancy when returned to prune agar may be so obtained. On the other hand, efforts to cause the stock strain to vary by changing the medium have not yielded new forms; what changes have been seen are due to the medium alone, and they are not retained in subcultures made on prune agar. This suggests that the changes obtained in the variants by a change of medium are to be ascribed, not directly to the medium, but to the unstable nature of the variants themselves, and so to the effect of the original treatment. Moreover, variants have been obtained on prune agar from heated spores taken from a line of cultures kept on that medium for years; it is evident that a change of medium is not necessary in order to bring about a change in the behaviour of the fungus. The uniform behaviour of the stock material in culture does not support a suggestion that the variants have arisen from peculiar spores which have been selected by the methods used in the work. If the strain normally produces spores able to give unusual colonies, some of the stock cultures should have borne variants derived from such spores.

It might be objected that the variants are merely contaminations selected from foul cultures, but this simple explanation cannot be accepted. Most of the work has been carried out on prune agar, and the strain of *E. herbariorum* has been grown in the laboratory on that medium for six years. Other strains of the same species have been grown on prune agar by other workers in the laboratory for the past eighteen years. There is no evidence that variant forms like those now under consideration have appeared in any of the hundreds of stock cultures prepared during this time. Further, for the past six years, dishes have been exposed from time to time, in order to determine what fungi could be obtained in the laboratory from infected dishes, and, until the present work was undertaken, none of the forms of *Eurotium* described in the present communication is known to have been present in the laboratory. Their appearance in cultures begins with the preparation of cultures from heated spores, and in all cases a new form has first appeared in a culture derived directly or indirectly from heated spores. Control cultures have been prepared from the stock material as a part of every experimental set of cultures, and a variant form has not been seen in any control.

Control cultures and stock cultures have remained throughout of normal type; cultures set up in parallel with them, in dishes poured from the same lot of medium, but inoculated with heated spores, have yielded aberrant colonies. Except for the preliminary heating of the spores, all the conditions have been the same for controls and for experimental cultures. It is difficult to ascribe the origin of new forms in this work to anything but the effects of heat on the spores.

A search has been made in the literature, in the hope that evidence might be found bearing on the suggestion that variation may be induced in fungi by heating the spores before they are sown. Two cases have come to light: Haenicke (10) records that a permanent change was caused in *A. niger* by shaking spores in agar at 50° C., and then pouring plates from the suspension. Blochwitz (3) obtained a new form of *A. versicolor*, distinguished by its blue conidia, in three cultures out of a number prepared from a stock culture. He stated that these three cultures, which contained normal colonies as well as the new form, were probably set up from the same charge of spores, the transfer being made with a platinum wire. Fresh cultures prepared from the parent stock failed to yield any blue colonies. In the light of the present work, it seems possible that the wire used by Blochwitz in the three significant transfers was hot, and that this caused the variation.

The variations which have been obtained in *E. herbariorum* may be arranged in the following order:

1. Young colonies, abnormal in form and in colour, but ultimately giving colonies not differing from normal.

2. Young colonies, distinctly abnormal, giving older colonies differing slightly from normal in the colour of the conidia, amount of aerial mycelium, density of growth, and increased formation of perithecia.

3. Distinct variant forms producing conidia and perithecia, but differing throughout their life from normal, in the form of the colony, density of growth, and coloration of the hyphae. This class includes flame and its derivatives.

4. Distinct variant forms producing conidia and perithecia. In C yellow both conidiophores and perithecia appear liable to deformity; in speckled, the perithecia are enveloped in Hulle cells.

5. Distinct variant forms giving conidia only; in the creamy variant, the yellow or orange colours which sometimes appear may be due to the presence of the pigment which accompanies the formation of perithecia in the normal form; in D brown the perithecial tendency is suggested by the abundant formation of Hulle cells.

This scheme, taken in conjunction with the descriptions of the variants

already given, suggests a distinct correlation between the degree of modification and the stability of the new forms; the less modified forms arise from slightly heated spores, the more altered forms from spores exposed to high temperatures. Classes 1 and 2 have been little investigated; what has been done indicates that these slightly divergent forms tend soon to revert to normal. In Class 3 stability is not of a high grade, and variations have been caused by a change of medium; transfers have also given cultures which fall into Class 2. Classes 4 and 5 include the most stable variants found. They have shown variation within narrow limits, and sectoring in C yellow has restored the normal form, but they do not appear to be specially sensitive to changes in the medium, and, with the exception noted, have not tended to revert.

Slow chemical changes must occur in the spore, even in the resting condition, as a result of respiration. Henderson Smith (12) has shown in *Botrytis cinerea* that the rate of chemical change in the spore is greatly increased by exposure to moderate rises of temperature; experiments on this matter have not been carried out with *E. herbariorum*, but there can be little doubt that the same considerations apply to it. Blochwitz (3) stated that the pigmentation of colonies of *Aspergillus* grown from old spores might differ from that shown by normal colonies. A colourless strain of *A. glaucus* was obtained by him from a culture nine years old: this strain had lost both the yellow pigment of the walls and the blue pigment of the hyphal contents. As the result of these and other observations, Blochwitz concluded that variations in fungi in culture could often be attributed to a derivation from old spores. It does not seem unreasonable to suggest that the changes in old conidia are due to the progress of slow respiration over a long period, and this consideration, taken in conjunction with the work of Henderson Smith (12), affords a clue to the possible nature of the changes observed in the present investigation. Sudden exposure to a high temperature will greatly increase the rate of change in the conidium, and may give in a short time as much modification as respiration could produce in several years. The changes may become apparent in the pigmentation of the colony obtained from the altered spore. Indications have been seen that other changes occur, in addition to the obvious and readily noted colour reactions. Reference has been made to the staining of the medium, and in the course of the small amount of morphological work which has been done, frequent examples have been noted of branching and proliferation of the conidiophores. The formation of giant cells by C yellow, and the production of colonies of irregular outline by flame and by C yellow, are also morphological changes, and ascospores without the furrows and frills usual in *Eurotium* have been obtained in Series A. The formation of the conidial apparatus has been delayed by growing the variants on Czapek's medium, and has been apparently suppressed altogether in a few cultures

of creamy. Thus, most of the characters of the fungus have been found liable to modification.

It has been pointed out in the descriptions of the series of cultures that early cultures in the series were usually dense in growth, and that gradual loosening and slow approximation to the spreading habit of *Eurotium* have been noticed in later cultures. This suggests that as culture has succeeded culture, slow change has taken place towards a condition of greater equilibrium than was present in the colonies obtained from heated spores, or from their immediate progeny. Further, the classification of the variants which has been given suggests a connexion between the steepness of the temperature gradient to which the spores have been exposed and the extent of the change in colonies they give. It is possible that the greatest variation was obtained from spores which had narrowly escaped death.

The strain of *E. herbariorum* which has been used has been in culture, at a moderately high temperature, for six years. This long period of culture may be partly responsible for the ease with which the strain has yielded variants under experimental treatment. The effect of heat has not yet been tried on uncultured material of unknown history.

The development of abnormal colonies from heated spores suggests that nuclear changes have been caused by the heating. The work of Blakeslee (1) and of Burgeff (6) on neutral strains of *Phycomyces nitens* indicates that changes in morphology and pigmentation may occur when nuclei of more than one kind are associated in the same mycelium. It is unlikely that all the nuclei of a multinucleate conidium will be changed to the same extent when the conidium is heated. The mycelium derived from the conidium will presumably contain nuclei of more than one kind, and this condition may be assumed to account for the alterations in form and colour which have been observed; this, however, remains to be demonstrated.

The systematic aspects of the problem can only be dealt with after a morphological study of the variants has been completed. For the present they are all regarded as coming within the range of *E. herbariorum*, and are compared with the numerous horticultural varieties of many cultivated plants. It may be suggested tentatively that this work, like that of Brooks and Hansford (4) on *Cladosporium*, of Stevens and Hall (14) on a number of fungi, of Blakeslee (2) on *Mucor*, and of Stevens (13) on *Helminthosporium*, indicates that the systematics of these variable forms may need revision. If variation may be caused in the laboratory by exposing spores to heat, there seems no reason to deny that under natural conditions strong insolation, accidental fires, or even the garden bonfire, may not from time to time give rise to new variants. Whether or no such variants could persist in nature, in free competition, cannot be said; the variants described here seem to hold their own against the normal form in mixed cultures.

Variations in fungi may doubtless be caused in other ways than by heating the spores. It has not seemed necessary in this place to give a complete analysis of all the literature in which variations in fungi are recorded and described; much of it does not bear directly on the present issue, for few details are given in the papers of the methods used in making transfers of material in the preparation of new cultures. Consequently, it is impossible to judge whether at some time or other the variation recorded has started in the offspring from a heated spore.

Summary.

Two colonies of abnormal form appeared suddenly in 1926 in cultures of a strain of *E. herbariorum*, which had remained constant in culture for five years. There was reason to think that the aberrant colonies had arisen from spores which had been accidentally heated. Experiments were performed to discover if variation could be caused by heating the spores before sowing them. 242 cultures were set up from heated spores; in 134 of these, 292 abnormal colonies were obtained, with 644 normal ones; of the remaining 108 cultures, 58 yielded some aberrant forms, and 38 showed no growth. Variations were not observed in 43 control cultures, containing about 2,000 colonies, nor have any been seen in stock cultures on various media.

Series of cultures have been made from the two abnormal colonies which suggested the work, and from some of the variants obtained from heated spores. The new forms tend to maintain their characters when grown exclusively on prune agar, but some show an alteration in behaviour after a period of growth on a synthetic medium. There is evidence that the variants which differ greatly from normal are less sensitive to change of medium than those which show less divergence; the stock strain has shown no tendency to react to a change of medium.

The available evidence supports the view that the variant forms have arisen from heated spores. The methods employed are capable of producing variations in the strain of *E. herbariorum* used, but they do not enable the result of a given experiment to be foretold.

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Literature cited.

1. Blakeslee, A. F.: Zygospore Germinations in the Mucorineae. Annales Mycologici, iv, p. 1, 1906.
2. ——————: Mutations in Mucors. Year Book, Carnegie Inst., Washington, xii, p. 104, 1913.
3. Blochwitz, A.: Eine allgemeine Ursache spontaner Verlustmutationen bei Schimmelpilzen. (Vorl. Mitt.) Ber. d. Deutsch. Bot. Ges., xli, p. 205, 1923.
4. Brooks, F. T., and Hansford, C. G.: Mould Growths upon Cold-store Meat. Trans. Brit. Myc. Soc., viii, p. 113, 1923.
5. Brown, W.: Studies in the Genus *Fusarium*. IV. On the Occurrence of Saltations. Ann. Bot., xl, p. 223, 1926.
6. Burgeff, H.: Untersuchungen über Variabilität, Sexualität und Erblichkeit bei *Phycomyces nitens* Kunze. I. Flora, cvii, p. 259, 1914.
7. Eidam, E.: Zur Kenntnis der Entwickelung bei den Ascomyceten. Cohn's Beitr., iii, p. 377, 1883.
8. Fraser, H. C. I., and Chambers, H. S.: The Morphology of *Aspergillus herbariorum*. Annales Mycologici, v, p. 421, 1907.
9. Gwynne-Vaughan, H. C. I., and Barnes, B.: The Structure and Development of the Fungi. Cambridge, 1927.
10. Haenicke, A.: Vererbungsphysiologische Untersuchungen an Arten von *Penicillium* und *Aspergillus*. Zeitschr. f. Bot., viii, p. 225, 1916.
11. Ridgway, R.: Color Standards and Color Nomenclature. Washington, D.C., 1912.
12. Smith, J. Henderson: The Killing of *Botrytis cinerea* by Heat, with a Note on the Determination of Temperature Coefficients. Ann. Applied Biol., x, p. 335, 1923.
13. Stevens, F. L.: The *Helminthosporium* Foot-rot of Wheat, with Observations on the Morphology of *Helminthosporium* and on the Occurrence of Saltation in the Genus. Bull. Illinois State, Nat. Hist. Survey, xiv, p. 77, 1922.
14. Stevens, F. L., and Hall, J. C.: Variation of Fungi due to Environment. Bot. Gaz., xlviii, p. 1, 1909.
15. Thom, C., and Church, M. B.: The Aspergilli. London, 1926.
16. Wehmer, C.: Übergang älterer Vegetationen von *Aspergillus fumigatus* in 'Riesenzellen' unter Wirkung angehäufter Säure. Ber. d. Deutsch. Bot. Ges., xxxi, p. 257, 1913.

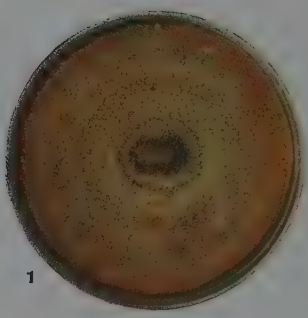
EXPLANATION OF PLATE XV.

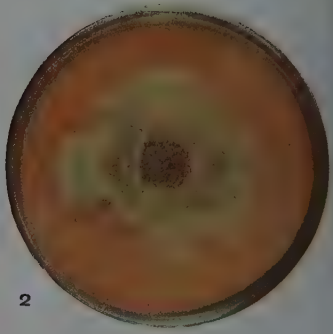
Illustrating Dr. Barnes's paper on *Eurotium herbariorum*.

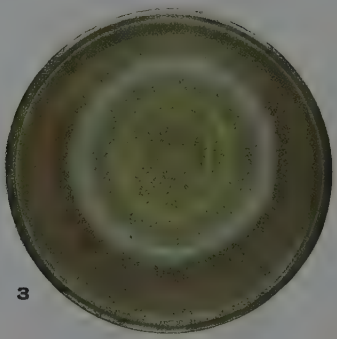
The colony shown in Fig. 7 was grown on Czapek's medium; the others on prune agar. All were incubated at 30° C.

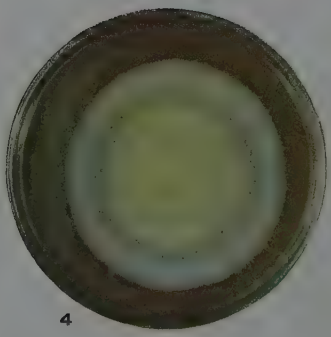
Fig. 1. A colony of the stock strain of *Eurotium herbariorum*.
Fig. 2. The flame variant.
Fig. 3. The green flame variant.
Fig. 4. The woolly flame variant.
Fig. 5. The blue conidial variant.
Fig. 6. The creamy variant.
Fig. 7. The creamy variant on Czapek's medium.
Fig. 8. The speckled variant.
Fig. 9. The D brown variant.
Fig. 10. The creamy variant from Series A, showing marked staining of the medium.

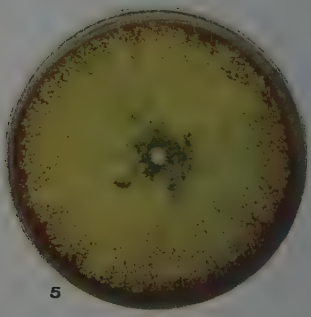
The colour of the medium in Figs. 3, 4, and 7 is darkened by the background against which they were photographed. In Fig. 10 the surface of the medium shows wrinkles due to drying.











C. E. B. Dobb, Col. Phot.

BARNES—EUROTIUM AND HIGH TEMPERATURE.

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